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FIRST FINDING OF TRIPLOID HYBRID FROGS *PELOPHYLAX ESCULENTUS* (ANURA: RANIDAE) IN MOZH RIVER BASIN (KHARKIV REGION, UKRAINE)

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Pelophylax esculentus is an interspecies hybrid of marsh frog *P. ridibundus* and pool frog *P. lessonae.* The hybrids are usually presented by diploid and triploid forms, and coexist and crossbreed with one or both parental species in the hemiclonal population systems (HPS). Siverskyi Donets river basin is known for its diversity of HPS and was described as Siverskyi Donets center of water frogs diversity. Three subregions were described within it based on the HPS composition features (diploid R-E, triploid-containing R-E-Ep and R-Epf with triploid females only among hybrids). The presence of triploid *P. esculentus* was earlier confirmed only for two of three subregions of the Siverskyi Donets river basin, while the third, R-E subregion (including Mozh river) was thought to be inhabited by only diploids. Here we present the results of analysis on ploidy and genome composition of both adult and juvenile water frogs in a pond in the Tymchenky village (Mozh river basin, Kharkiv region, Ukraine). Three samples of frogs were collected in September 2019, June 2020, and August 2021 (109 adults and 56 juveniles in total) and analyzed using microscopic erythrocyte cytometry (dry smears), karyology of bone marrow, fluorescent staining (with DAPI) and analysis of morphological features. We identified 2 triploid males among adults and 5 triploids of both sexes among juveniles. The overall triploid ratio between ages changed drastically (9% among juveniles vs 1% among adults), but insignificantly (p=0.078). The erythrocyte size indicating margin between adult di- and triploids was established as 28 μm for this system; for juveniles such margin is quite unclear. All triploids had genome composition LLR (i.e. two genomes of *P. lessonae* and one genome of *P. ridibundus*). By the majority of diploid *P. esculentus* and the presence of triploids, Tymchenky system appeared similar to some HPSs (Koriakiv, Iskiv systems) in other subregions, known for triploid presence. The presence of triploids, contrary to previous data on this region, may be explained by several hypotheses: (1) rare observation of triploids; (2) migration of either triploids or *P. esculentus* producing 2n-gametes; (3) a newly evolved feature of local *P. esculentus* reproduction.
Key words: *Pelophylax esculentus, Pelophylax ridibundus, hemiclonal population system, hybrid, triploid*

INTRODUCTION

European edible frog (*Pelophylax esculentus* L., 1758), a hybrid of pool frog (*Pelophylax lessonae* Camerano, 1882) and marsh frog (*Pelophylax ridibundus* Pallas, 1771), is well-known by the phenomenon of hemiclonal reproduction [6-8; 11; 12; 18; 25]. It means transmitting parental genome(s) clonally, with hybrids reproducing in crosses with parental species or between each other. *P. esculentus* typically coexist with one or both parental species in mixed populations, which are called hemiclonal/hybridogenetic population systems (HPS) [e. g. 11; 14; 18; 19; 21; 23; 24]. Considering the variety of gametes that hybrids can possibly produce (L, R, LL, RR, LR, and a mixt of several types), all members of population systems are involved in the reproduction of each other, and each system evolves as a whole.

Previous studies on hemiclonal population systems in Eastern Ukraine resulted in a description of Siverskyi Donets center of water frogs diversity – the region with a huge variety of population system types along with the paradoxical absence of pool frogs [e. g. 2; 5; 8; 9]. All the variety of population systems were classified according to their composition and, thus, evolutionary peculiarities: 1) R-E-HPS – systems with marsh frog (denoted by R) and 2n-hybrids (E); 2) R-E-Ep-HPS – similar systems, but with 3n-polyploids too (Ep); 3) R-

Epf-HPS – systems with marsh frogs and only triploid female hybrids (Epf) (Fig. 1). The whole diversity of the center is considered to be associated with the biggest R-E-Ep subregion, occupying the forest-steppe area of Siverskyi Donets drainage. North-western Siverskyi Donets tributaries and its downstream belong to different and simpler subregions: R-E and R-Epf respectively. The geographic distribution of these subregions supposedly reflects the evolutionary ways within the diversity center [21].

Since the evolution of water frogs appears connected to population system distribution, any shifts of existing sub-regions are especially interesting. Hereafter we report the early unknown R-E-Ep system (beyond the R-E-Ep subregion) in the Mozh River basin, revealed during annual water frogs monitoring. This area was assigned to the R-E (diploid) region due to morphology, DNA-cytometry (2010-2012 years, in collaboration with S. Litvinchuk and Yu. M. Rosanov [22]), and karyology (2016-2018) analyses [2; our not published data], performed on the set of small samples of hybrid individuals.

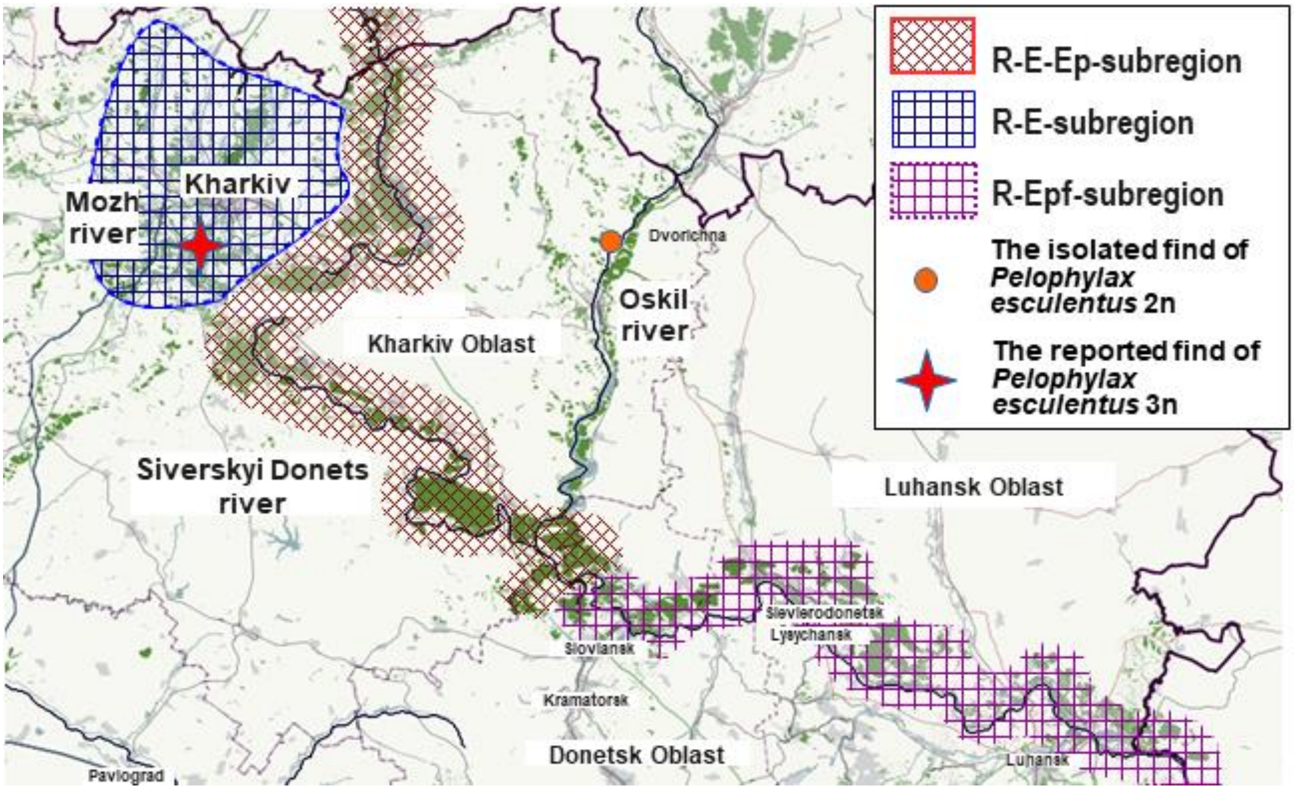

Fig. 1. Previously described Siverskyi Donets diversity center typology and registered new locality of "triploid-inhabited" HPS (red star). The map was taken from [21] with modifications.

MATERIALS AND METHODS

Three samples of frogs were collected in September 2019, June 2020, and August 2021 in the spring-fed artificial pond (30×35 m), situated on the sand terrace of Mozh River (Tymchenky village, Kharkiv region, Ukraine; 49.7492, 36.1629). Frogs were hand-caught at night using a flashlight. Taxonomical and sex identification for adults was carried out by external morphology [18; 22; 23]. Juvenile morphology is not reliable enough, so their species identification was only preliminary, and sex was identified by gonadal morphology [13]. SVL (snout-vent length) was measured for all individuals using calipers. Ploidy was roughly estimated by the average erythrocyte size measured on dry blood smears [4; 17]. For all the juveniles and adults with the biggest erythrocytes, karyological analysis of intestine epithelium and bone marrow was performed [3 with modifications]. We identified ploidy by mitotic chromosome number: 2n=26, 3n=39. Genome composition of triploid hybrids was established using DAPI-staining (combined method of [16] and [20] with modifications). All slides were inspected using a Leica DM 2000 microscope equipped with standard fluorescence filter sets (for fluorescently stained slides we used "DAPI 390" filter cube). Microphotographs were captured with a Leica DFC3000 G camera using Leica LASX Software. To compare different metrics between groups, a non-parametric Mann-Whitney U-test was used; to compare the distributions of different forms between ages or systems the Chi-square test was applied. Statistical and descriptive analysis was performed using R.

Pelophylax species are not listed in the Red Data Book of Ukraine and listed by the IUCN Red list as "least concerned". All specimens were collected in the region of Ukraine, which is not considered a protected area. Techniques used in the capture, tissue sampling, and euthanasia sought to minimize animal suffering according to Directive 2010/63/EU on the protection of animals used for scientific purposes.

RESULTS

The total individuals caught were 109 adults and 56 juveniles; species, sex, and ploidy distribution are presented in Table 1. The sample of the 2019 year was taken in autumn and consisted of juveniles only; the sample of the 2020 year was taken during the breeding season and thus contained only adults; the sample of the 2021 year was taken in late summer so had both ages.

Considering the unconfident species identification for diploid juveniles, the species ratios were not compared between ages. The overall triploid ratio between ages changed drastically (9% among juveniles vs 1% among adults), but insignificantly ($p = 0.078$). Via DAPI-staining, we identified 5 juveniles (3 males, 2 females) and 2 adult males as triploids with genome composition LLR. The other form of triploids, LRR, was not found.

Table 1.

Species, sex, ploidy, and age of analyzed water frogs by years. Dash line denotes the absence of correspondence age in some samples; zero denotes the forms which were not observed.

Form of water frog			Year of sample		
Ploidy	Species	Sex	2019	2020	2021
Diploid (2n)	*P. esculentus*	Male	–	43	47
		Female	–	0	1
	P. ridibundus	Male	–	0	3
		Female	–	1	12
	Juveniles (undefined species)	Male	18	–	10
		Female	5	–	18
Triploid (3n)	*P. esculentus*	Male	–	2	0
		Female	–	0	0
	Juveniles (*P. esclulentus*)	Male	2	–	1
		Female	2	–	0
		Overall	27	46	92

Whereas the females were the largest animals, no significant differences by SVL among groups of adults were found. But among diploid juveniles, males had a higher snout-vent length ($p = 0.004$).

Morphology and erythrocyte measuring are the classical methods to distinguish hybrids and triploids within them. But they appeared to be reliable methods for adult frogs only, while our data show that it fails while studying immature individuals. For example, among 8 juveniles, primary (morphologically) identified as *P. ridibundus*, 2 revealed to be triploid *P. esculentus*. Juveniles also demonstrated a wider erythrocyte size range (17.81-28.48 µm for 2n; 20.23-32.01 µm for 3n) than adults did (21.02-27.4 and 29.07-30.9 µm, respectively) (Fig. 2). We found significant differences by mean erythrocyte length between ages, but only for diploids ($p<0.0001$), while for triploids it was not significant (possibly due to a small number of triploids). Juveniles erythrocyte lengths also differed between sexes (both among diploids, $p = 0.002$, and all together $p = 0.019$, but not among triploids).

All this would definitely make it hard to predict the actual triploidy-margin erythrocyte size for this population system if we used juveniles only. For adults, this margin was identified as 28 µm; for other systems, it can be different (e.g. 26 µm [3]).

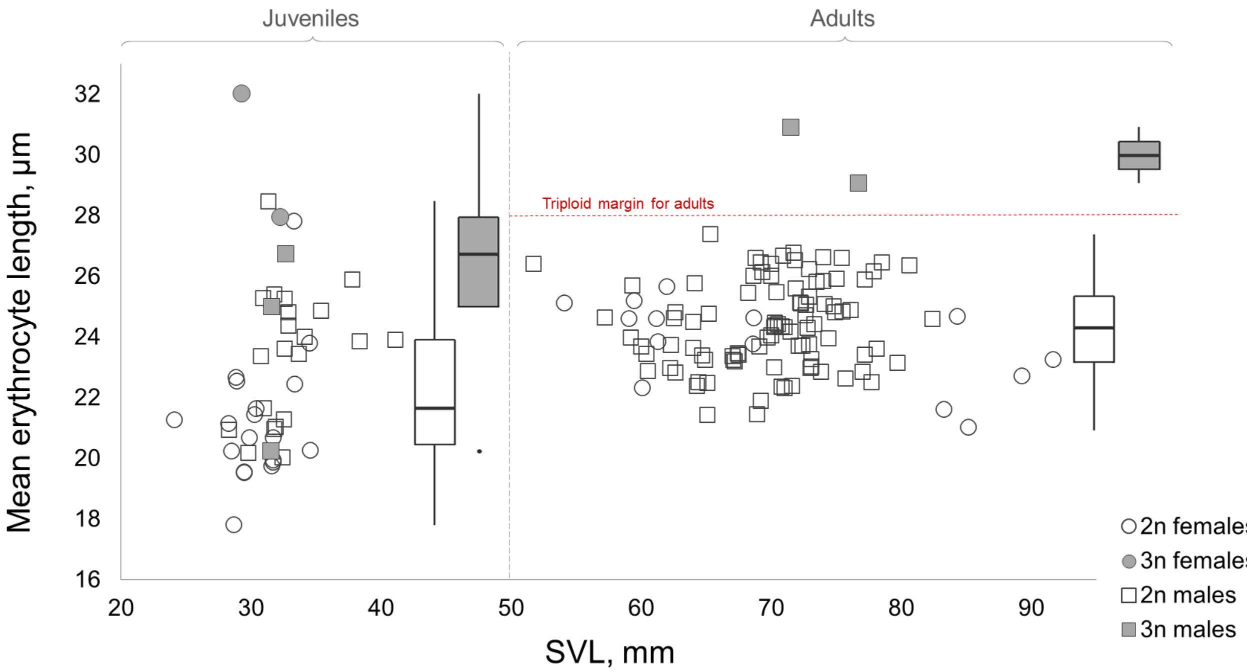

Fig. 2. Distribution of analyzed individuals by mean erythrocyte size and sex (scatterplot), and erythrocyte size distribution for each ploidy level (boxplots for each age group). Boxplots denote median (central line), first and third quartiles (box hinges), and extreme values no more than 1.5xIQR from the box (whiskers). The horizontal dotted red line denotes "triploids margin", defined for adults in this system as ~28 µm.

DISCUSSION

Here we show the presence of triploid hybrid water frogs *P. esculentus* in the Mozh river basin for the first time. We suggest a set of scenarios for a hypothetical explanation of this phenomenon:

1) recent expansion of R-E-Ep region via migration of water frogs (especially *P. esculentus*, producing 2n-gametes) upstream of Mozh river;

2) recent evolving of the ability to produce 2n-gametes by the local *P. esculentus* lineages;

3) long time existence of triploid *P. esculentus* in Tymchenky HPS unnoticed.

The third scenario seems to be the most possible: all the previously studied samples from the Mozh river basin were not numerous. Considering the fact that the portion of triploids in the Tymchenky pond was rather small, they may simply not be found in previous samples. Such a scenario implies the possibility of existence (or regular emergence) of triploids in other diploid systems (R-E-HPS) at low amounts, previously undetected. Therefore, other systems in the R-E region need reinspection.

Tymchenky system appeared to have roughly the same portion of triploid hybrids as two other already known systems in for Siverskyi Donets center: Iskiv (49.6278, 36.2828) and Koriakiv pond (49.6159, 36.3114). These systems also contain a massive portion of diploid hybrids with low but regularly observed triploids (particularly due to their long-term monitoring) [1; 10; 15]. Ratio of frogs with different ploidy in both Koriakiv and Iskiv systems is similar to the studied system in Tymchenky (chi-square significance levels were p = 0.438 and p~1 respectively) (Fig. 3). However, those two systems are known to contain LRR triploids also, which indicates their principal distinctiveness from Tymchenky HPS.

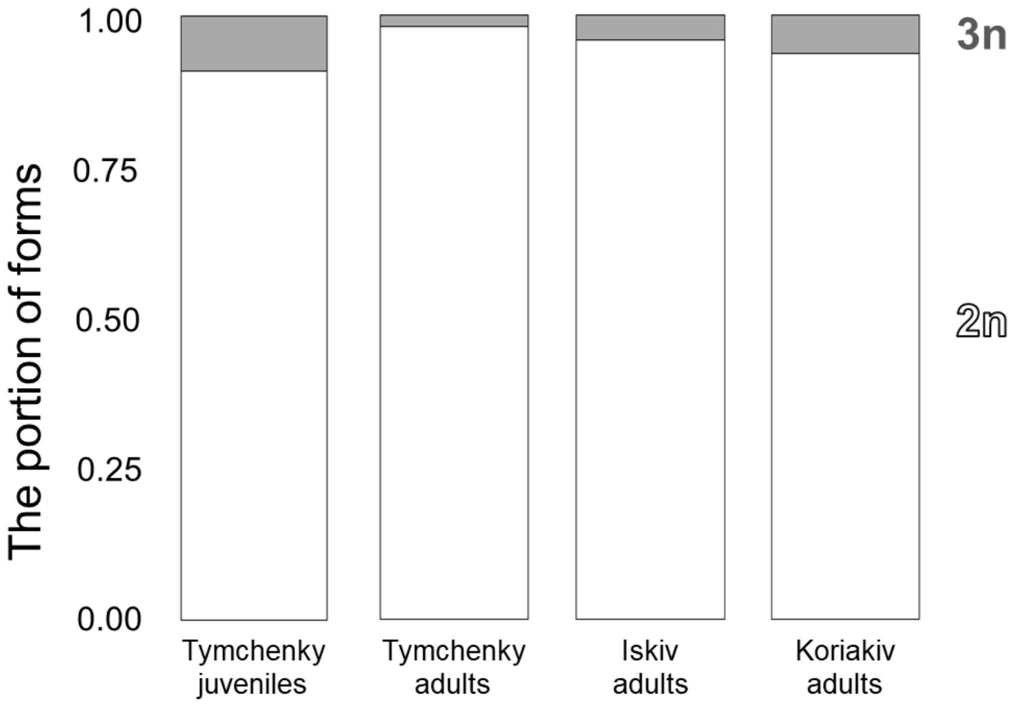

Fig. 3. Portions of frogs with different ploidy in the Tymchenky system by ages, and in Iskiv and Koriakiv systems.

CONCLUSION

Regarding all the data obtained since 2010, the Tymchenky population system contains *P. ridibundus*, diploid *P. esculentus*, and LLR-triploid *P. esculentus* of both sexes – thus it should be described as an R-E-Ep-HPS. The presence of triploids is contrary to previous data on this region [21] and may imply either rare triploid observation, migration of triploids, or *P. esculentus* producing 2n-gametes, or a newly evolved feature of local *P. esculentus* reproduction. Further exploration of the R-E system (particularly in the Mozh River basin) is apparently perspective and necessary for studying the *Pelophylax esculentus* complex evolution.

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ПЕРША ЗНАХІДКА ТРИПЛОЇДНИХ ГІБРИДНИХ ЖАБ *PELOPHYLAX ESCULENTUS* (ANURA: RANIDAE) В БАСЕЙНІ РІЧКИ МОЖ (ХАРКІВСЬКА ОБЛ., УКРАЇНА)

Дрогваленко М., Пустовалова Е., Федорова А., Шабанов Д.

Pelophylax esculentus – міжвидовий гібрид озерної жаби *P. ridibundus* та ставкової жаби *P. lessonae*. Гібриди зазвичай представлені диплоїдними і триплоїдними формами, які співіснують і схрещуються з одним або обома батьківськими видами в геміклональних популяційних системах (ГПС). Басейн річки Сіверський Донець відомий своїми різноманітними ГПС і був описаний як Сіверсько-Донецький центр різноманіття зелених жаб. У його межах було описано три субрегіони на основі особливостей складу ГПС (диплоїдні – R-E, з триплоїдами – R-E-Ep і R-Epf лише з триплоїдними самками серед гібридів). Наявність триплоїдних *P. esculentus* раніше була підтверджена лише для двох із трьох субрегіонів басейну річки Сіверський Донець, тоді як третій, R-E-субрегіон (включаючи р. Мож), вважався населеним лише диплоїдами. В даній статті ми представляємо результати аналізу плоїдності та складу геному як дорослих зелених жаб, так і цьогорічків у ставку в с. Тимченки (басейн р. Мож, Харківська область, Україна). Три вибірки жаб були зібрані у вересні 2019, червні 2020 та серпні 2021 року (всього 109 дорослих особин і 56 молодих особин) та проаналізовані за морфологічними особливостями, а також з використанням методів цитометрії еритроцитів (сухі мазки), каріології кісткового мозку та флуоресцентного фарбування (за допомогою DAPI). Ми виявили 2 триплоїдних самці серед дорослих і 5 триплоїдів обох статей серед цьогорічків. Загальне співвідношення триплоїдів за віком різко змінюється (9% серед цьогорічків проти 1% серед дорослих), але незначуще (p=0,078). Розмір еритроцитів, що вказує на межу між дорослими ди- і триплоїдами, був встановлений як 28 мкм для цієї системи; для цьогорічків така межа не є очевидною. Усі триплоїди мали склад геному LLR (тобто два геноми *P. lessonae* та один геном *P. ridibundus*). За більшістю диплоїдних *P. esculentus* та наявністю триплоїдів система у Тимченках виявилася схожою на деякі інші ГПС (системи Корякова та Іськова ставків) в інших субрегіонах, які характеризуються присутністю триплоїдів. Наявність триплоїдів, всупереч попереднім даним щодо цього регіону, може пояснюватись декількома гіпотезами: (1) рідкісні знахідки триплоїдів; (2) їх міграція чи міграція *P. esculentus*, які продукують 2n-гамети; (3) нова особливість розмноження *P. esculentus*, що виникла нещодавно.

Ключові слова: *Pelophylax esculentus, Pelophylax ridibundus, геміклональна популяційна система, гібрид, триплоїд*